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MicroReview

The lipid A palmitoyltransferase PagP: molecular mechanisms and role in bacterial pathogenesis

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Summary

Palmitoylated lipid A can both protect pathogenic bacteria from host immune defences and attenuate the activation of those same defences through the TLR4 signal transduction pathway. A palmitate chain from a phospholipid is incorporated into lipid A by an outer membrane enzyme PagP, which is an 8-stranded antiparallel β-barrel preceded by an amino-terminal amphipathic α-helix. The PagP barrel axis is tilted by 25° with respect to the membrane normal. An interior hydrophobic pocket in the outer leaflet-exposed half of the molecule functions as a hydrocarbon ruler that allows the enzyme to distinguish palmitate from other acyl chains found in phospholipids. Internalization of a phospholipid palmitoyl group within the barrel appears to occur by lateral diffusion from the outer leaflet through non-hydrogen-bonded regions between β-strands. The MsbA-dependent trafficking of lipids from the inner membrane to the outer membrane outer leaflet is necessary for lipid A palmitoylation *in vivo*. The mechanisms by which bacteria regulate *pagP* gene expression strikingly reflect the corresponding pathogenic lifestyle of the bacterium. Variations on PagP structure and function can be illustrated with the known homologues from Gram-negative bacteria, which include pathogens of humans and other mammals in addition to pathogens of insects and plants. The PagP enzyme is potentially a target for the development of anti-infective agents, a probe of outer membrane lipid asymmetry, and a tool for the synthesis of lipid A-based vaccine adjuvants and endotoxin antagonists.

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Introduction

Lipid A is the hydrophobic anchor of lipopolysaccharide (LPS), which forms the outer leaflet of the outer membrane (OM) of Gram-negative bacteria (Raetz and Whitfield, 2002). Lipid A is an essential structural component of the Gram-negative bacterial cell envelope and constitutes a target for the development of antibacterial agents. Some non-essential modifications to the structure of lipid A have proven necessary for bacterial virulence and, thus, provide targets for anti-infective agents (Trent, 2004; Bishop, 2005). Lipid A is also the active component of LPS endotoxin, which can promote septic shock when shed from the bacterial surface during systemic infection (Miller *et al*., 2005). This inflammatory reaction is mediated through the Toll-like receptor 4 (TLR4) signal transduction pathway, and leads to the production of cytokines, chemokines and antimicrobial agents needed to mount an effective innate immune response to bacterial infection. Additionally, activation of TLR4 by lipid A initiates the expression on antigen-presenting cell surfaces of the costimulatory molecules needed to mount an adaptive immune response to infection (Iwasaki and Medzhitov, 2004). The inflammatory response is a double-edged sword because it is necessary to eliminate most infections and, at the same time, responsible for some of the main pathophysiological symptoms associated with persistent infections. Bacterial pathogens and symbionts can coexist with their hosts in part because they modify the structure of lipid A to attenuate the inflammatory response and evade immune recognition. Modified lipid A structures are currently being developed for use both as endotoxin antagonists to treat septic shock and as vaccine adjuvants (Persing *et al*., 2002; Hawkins *et al*., 2004). Here, I review recent progress in the investigation of a single lipid A modification, namely, lipid A palmitoylation. Independent lines of study focused either on the biochemistry of the responsible enzyme or on the role of its corresponding gene in bacterial pathogenesis are now converging to make testable predictions about the molecular mechanisms of infectious disease.

Attenuation of endotoxin signalling

The constitutive lipid A endotoxin that is found in most enterobacteria is a β-1′,6-linked disaccharide of glucosamine, which is phosphorylated at the 1 and 4′ positions and acylated with primary *R*-3-hydroxymyristate chains at the 2, 3, 2′ and 3′ positions. The structure is further acylated with secondary laurate and myristate chains in acyloxyacyl linkage at the 2′ and 3′ positions respectively (Fig. 1). This hexa-acylated lipid A species can be modified by the regulated addition of a palmitate chain in acyloxyacyl linkage at position 2 to produce a hepta-acylated lipid A species (Bishop *et al*., 2000). Studies of immune signalling in human cell lines using chemically synthesized hexa-acylated and hepta-acylated lipid A revealed that palmitoylation attenuates the response to lipid A by 10- to 100-fold (Loppnow *et al*., 1986; Feist *et al*., 1989). One study indicated that palmitoylation converts lipid A into an endotoxin antagonist because prior treatment of human monocytes or macrophages with hepta-acylated lipid A could block signalling by the hexa-acylated molecule (Tanamoto and Azumi, 2000; Muroi *et al*., 2002). On its own, hepta-acylated lipid A was inactive as a signalling molecule in these studies. However, efforts to reproduce these results only served to reinforce the earlier conclusion that palmitoylation merely attenuates lipid A signalling (Janusch *et al*., 2002). The authors suggest that variations in the levels of expression of TLR4 and/or its associated factor MD-2 might explain these distinctly different observations. Similar conclusions that palmitoylated lipid A is less active were recently obtained using the corresponding natural lipid A species, which were isolated from bacteria that possessed key lipid A-modifying enzymes (Kawasaki *et al*., 2004). Palmitoylated lipid A normally coexists as a substoichiometric component with other regulated covalent lipid A modifications, which together can directly benefit the bacterium independently of any effects they might have on lipid A signalling.

Antimicrobial peptide resistance

The *pagP* gene responsible for lipid A palmitoylation was first identified in a *Salmonella* mutant that was found to be sensitive to cationic antimicrobial peptides (CAMPs), which are included among the products of the TLR4 signal transduction pathway (Guo *et al*., 1998; Levy, 2004). Consequently, the modification of lipid A with a palmitate chain appears, remarkably, to both attenuate the production of CAMPs through the TLR4 pathway and protect the bacterial pathogen from the antimicrobial products of that same pathway. However, it is important to recognize that

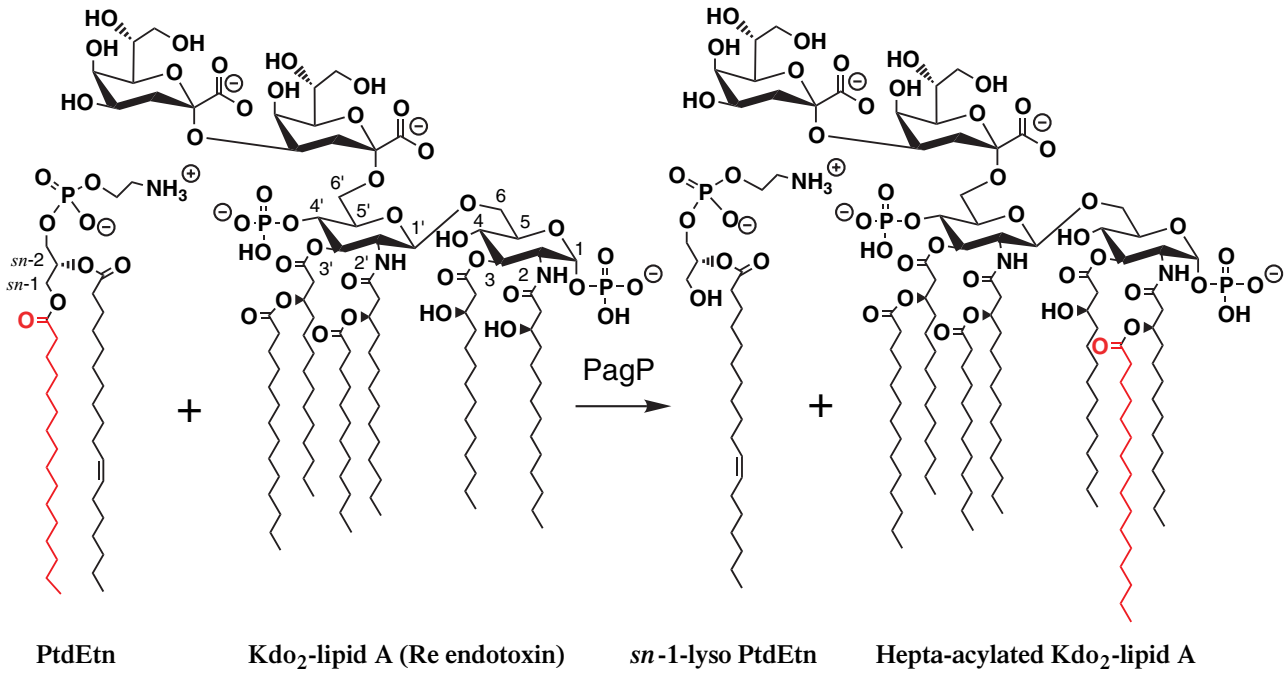

Fig. 1. PagP catalysed transfer of a palmitate chain from the *sn*-1 position of phosphatidylethanolamine (PtdEtn) to the simplest form of *E. coli* LPS (Re endotoxin), which consists of lipid A linked at the 6′ position with two units of 3-deoxy-D-*manno*-2-octulosonic acid (Kdo). Lipid A is a β-1′,6-linked disaccharide of glucosamine that is acylated with *R*-3-hydroxymyristate chains at the 2, 3, 2′ and 3′ positions, and phosphorylated at the 1 and 4′ positions. Acyloxyacyl linkages with laurate and myristate chains at the 2′ and 3′ positions, respectively, provide the constitutive hexa-acylated lipid A, which is a potent endotoxin. A regulated proportion of lipid A in *E. coli* contains a palmitate chain (16:0) in acyloxyacyl linkage at position 2, which yields a hepta-acylated molecule with attenuated endotoxic properties.

pagP only provides resistance to certain CAMPs, not all of which are products of the innate immune system (Guina *et al*., 2000). CAMPs are generally unstructured in aqueous solution and encounter bacteria by electrostatic interactions with the negatively charged bacterial surface (Hancock *et al*., 1995). The increased hydrophobicity at the membrane interface strips hydrogen bonded water molecules from the peptide bonds to drive folding into α-helical or β-sheet conformations. The induced secondary structure reveals the amphipathic nature of CAMPs, which have opposing polar and hydrophobic faces (Epand *et al*., 1995). The amphipathic structure facilitates the insertion of CAMPs into the hydrocarbon domain of the OM before uptake into the periplasmic space. Ultimately, CAMPs target the inner membrane, where they kill bacterial cells primarily by disrupting the membrane potential (Zasloff, 2002). However, the OM provides a significant barrier to CAMP uptake, and lipid A palmitoylation presumably increases the hydrophobic and van der Waals interactions in the OM so as to interfere with CAMP translocation across the bilayer. Neutralization of negative charges in lipid A by modification of the phosphates with positively charged L-4-aminoarabinose and/or phosphoethanolamine substituents similarly provides resistance to CAMPs, but in this case the mechanism involves blocking the initial electrostatic interactions (Trent, 2004). Only modification of the lipid A acylation pattern by palmitoylation is known to be additionally associated with attenuation of lipid A signalling through the TLR4 pathway (Kawasaki *et al*., 2004). Modification of lipid A by 3-O-deacylation similarly attenuates lipid A signalling, but without any other apparent functional consequences (Trent *et al*., 2001; Geurtsen *et al*., 2005). The functional importance of regulated S-2-hydroxylation at the secondary myristate chain is also unclear (Gibbons *et al*., 2000), but these two lipid A modifications each provide a hydrogen bond-donating hydroxyl group that might help to stabilize lateral interactions between neighbouring LPS molecules in the OM (Nikaido, 2003). Finally, one report indicates that resistance to certain CAMPs is associated with regulated lipid A dephosphorylation in *Salmonella* (Shi *et al*. 2004), but the molecular basis for this modification is uncertain given that known lipid A phosphatases appear to be absent from this organism (Price *et al*., 1995; Karbarz *et al*., 2003; Wang *et al*., 2004).

Mg^{2+} as a regulatory signal

The *Salmonella* PhoP/PhoQ two-component virulence signal transduction pathway regulates at least 40 genes, many of which are important for *Salmonella* virulence and lipid A modifications (Guo *et al*., 1997; Groisman, 2001). The PhoP/PhoQ-activated gene *pagP* is important both for CAMP resistance and palmitoylation of lipid A (Guo *et al*., 1998). The PhoQ signal transducer controls phosphorylation of the PhoP transcription factor in response to low extracellular Mg^{2+}-ion concentrations, which are likely encountered within the phagolysosomal compartment of macrophages (Garcia Vescovi *et al*., 1996). The requirement of Mg^{2+} to maintain OM lipid asymmetry by bridging negative charges in the LPS is recognized by the immune system as a bacterial 'Achilles' heel'. The natural resistance-associated macrophage protein (NRAMP1) pumps divalent cations out of the phagolysosome and into the cytosol in order to create an inhospitable environment for captured bacterial invaders (Forbes and Gros, 2001). CAMPs themselves can activate the PhoP/PhoQ system presumably by displacing Mg^{2+}-ions from the bacterial surface (Bader *et al*., 2003). Lipid A modifications likely serve as a bacterial defence mechanism against the antimicrobial strategies of the innate immune system and PhoP/PhoQ is needed for *Salmonella* bacteria to survive within macrophages. These observations suggest that PagP might play a role in the establishment of *Salmonella* infections within their hosts. However, *pagP* mutants of *Salmonella* are not compromised in terms of intracellular survival (Belden and Miller, 1994), suggesting that additional lipid A modifications are needed to produce the multiple lipid A molecular subtypes known to be present when these bacteria reside within the phagolysosome (Gibbons *et al*., 2005).

Laboratory strains of *Escherichia coli* possess a functional *pagP* homologue (*crcA*), but its level of expression is lower than that of its close relative in *Salmonella* (Bishop *et al*., 2000). While *E. coli* possesses enzymes for the addition of L-4-aminoarabinose, phosphoethanolamine and palmitate to lipid A, corresponding enzymes for 3-O-deacylation and S-2-hydroxylation do not appear to be present. Furthermore, it now appears that the lipid A-modifying structural genes shared between *E. coli* and *Salmonella* are not regulated in the same way. Part of this difference has been traced to the presence in *E. coli* of an upstream effector of PhoP/PhoQ known as EvgA/EvgS (named on the basis of a close similarity with *Bordetella* BvgA/BvgS) (Eguchi *et al*., 2004), and the absence in *E. coli* of a functional connection mediated by PmrD to the downstream effector of PhoP/PhoQ known as PmrA/PmrB (Winfield and Groisman, 2004). The PmrB signal transducer senses mildly acidic and/or high ferric iron conditions to control phosphorylation of the PmrA transcription factor, which regulates genes needed for the modification of lipid A phosphates with L-4-aminoarabinose and phosphoethanolamine substituents (Wosten *et al*., 2000; Gibbons *et al*., 2005). PmrD is the product of a PhoP/PhoQ-activated gene that can directly activate *Salmonella* PmrA by a post-translational mechanism (Kox *et al*., 2000). Interestingly, PmrD is absent from *Yersinia pestis*, but the connection between *pmrA*-dependent pro-

moters and the PhoP/PhoQ system has, nevertheless, been reinforced by the acquisition of *bona fide phoP* operator sequences (Winfield *et al*., 2005). Additionally, expression of a subset of *Salmonella* PhoP/PhoQ-dependent genes that are mostly absent from *E. coli* depends on a downstream transcription factor encoded by *slyA* (Navarre *et al*., 2005). Given that infection by *E. coli* proceeds from within the intestinal lumen, *Shigella* enters the cytoplasm of epithelial cells and *Salmonella* is taken up into phagolysosomes, it will be interesting to learn whether differences in *pagP* regulation correlate with these distinct pathogenic strategies.

Enzymology in the outer membrane

Efforts to elucidate the structure–function relationships of *E. coli* PagP were initially motivated by a desire to answer fundamental questions about lipid enzymology as it occurs in biological membranes. Despite their importance in cell signalling, the membrane-bound enzymes of lipid metabolism are poorly understood biochemically because they are notoriously difficult to purify from membranes in an active state (Vance and Vance, 2002). The lipid A palmitoyltransferase PagP is one of the few enzymes present in the OM of Gram-negative bacteria, and it displays particularly favourable biochemical properties including an intrinsic stability towards proteolytic digestion and heat denaturation, and the absence of cysteines (Bishop *et al*., 2000). PagP is normally produced at low levels and, like all OM proteins, it is targeted for export across the inner membrane by a cleavable amino-terminal signal peptide. Export *in vivo* limits the amount of protein that can be overproduced to about 1 mg l^{-1} of bacterial culture. PagP so produced can be readily detergent-solubilized from the OM and purified for enzymological studies in detergent micelles. For structural studies, the signal peptide is typically removed to allow PagP to accumulate in the cytoplasm as an insoluble aggregate that can be dissolved with chaotropic reagents and purified in quantities greater than 10 mg l^{-1} of bacterial culture. The denatured protein is then quantitatively refolded by rapid dilution into an appropriate detergent, where it remains stable for months at room temperature. These features and the monomeric nature of PagP are largely responsible for the fact that its structure and dynamics have been elucidated by both NMR spectroscopy and X-ray crystallography (Hwang *et al*., 2002; 2004; Ahn *et al*., 2004; Tugarinov *et al*., 2004; Hwang and Kay, 2005).

PagP transfers a palmitate chain from the *sn*-1 position of a phospholipid to the hydroxyl group of the *R*-3-hydroxymyristate chain at position 2 of lipid A in the OM (Fig. 1). The PagP protein is an 8-stranded antiparallel β-barrel preceded by an amino-terminal amphipathic α-helix (Ahn *et al*., 2004). The aromatic belts that define the membrane interfaces in OM β-barrel proteins only become evident when the PagP barrel axis is tilted by 25° (Fig. 2). A bound detergent molecule fixed normal to the membrane plane in the crystal structure reveals the active site, which is located inside the barrel in the outer leaflet-exposed half of PagP. The interiors of β-barrel membrane proteins are typically lined with polar amino acid side-chains but this is only true in the inner leaflet-exposed half of PagP, where nine interior water molecules are observed. The active site appears to require that phospholipids migrate by lateral diffusion from the membrane outer leaflet through non-hydrogen bonded zones between the β-strands. The β-barrel structure typically satisfies the hydrogen bonding requirements of peptide bonds in a membrane environment (Schulz, 2002), but in PagP, proline residues, which lack an amide proton to donate a hydrogen bond, disrupt the hydrogen bonding continuity at two positions in the outer leaflet-exposed half of the protein. A proline in β-strand A creates a β-bulge that both contributes to the highly dynamic nature of the large extracellular L1 loop and changes the registration of the hydrogen bonds that close the β-barrel (Hwang *et al*., 2002). Consequently, outer leaflet hydrogen bonds are missing between β-strands A and B, while those that close the barrel between β-strands A and H are sensitive to the detergent environment (Fig. 3). Four invariant prolines between β-strands F and G similarly disrupt the hydrogen bonding continuity in the outer leaflet, but, in this case, the structure of the β-strands themselves is not disturbed. The observed structural irregularities make the central cavity of PagP accessible to lipids in the OM outer leaflet.

Most phospholipid acyltransferases have a good degree of positional specificity for acyl chains in the glycerol backbone, but the particular type of acyl chain at a given position is usually discriminated only weakly. Any given phospholipid can be regarded as a small combinatorial library with respect to the distribution of various acyl chain types (Dowhan, 1997). In this regard, PagP is unusual in its ability to distinguish a 16-carbon saturated acyl chain, palmitate, from any other acyl chain, even those that differ by a single methylene unit (Brozek *et al*., 1987; Bishop *et al*., 2000). The concept of a hydrocarbon ruler was first proposed to account for the ability of some soluble acyltransferases to select a particular type of acyl chain (Wyckoff *et al*., 1998). The PagP active site provides the first structural evidence for a hydrocarbon ruler in an integral membrane enzyme. Substitution of Gly88 lining the floor of the hydrocarbon ruler with Ala, Cys and Met makes the enzyme selective for acyl chains shortened by one, two and four methylene units, respectively, indicating that hydrocarbon ruler depth determines acyl chain selection (Ahn *et al*., 2004).

Like most membrane-bound enzymes of lipid metabolism, PagP requires a detergent for activity. However, only

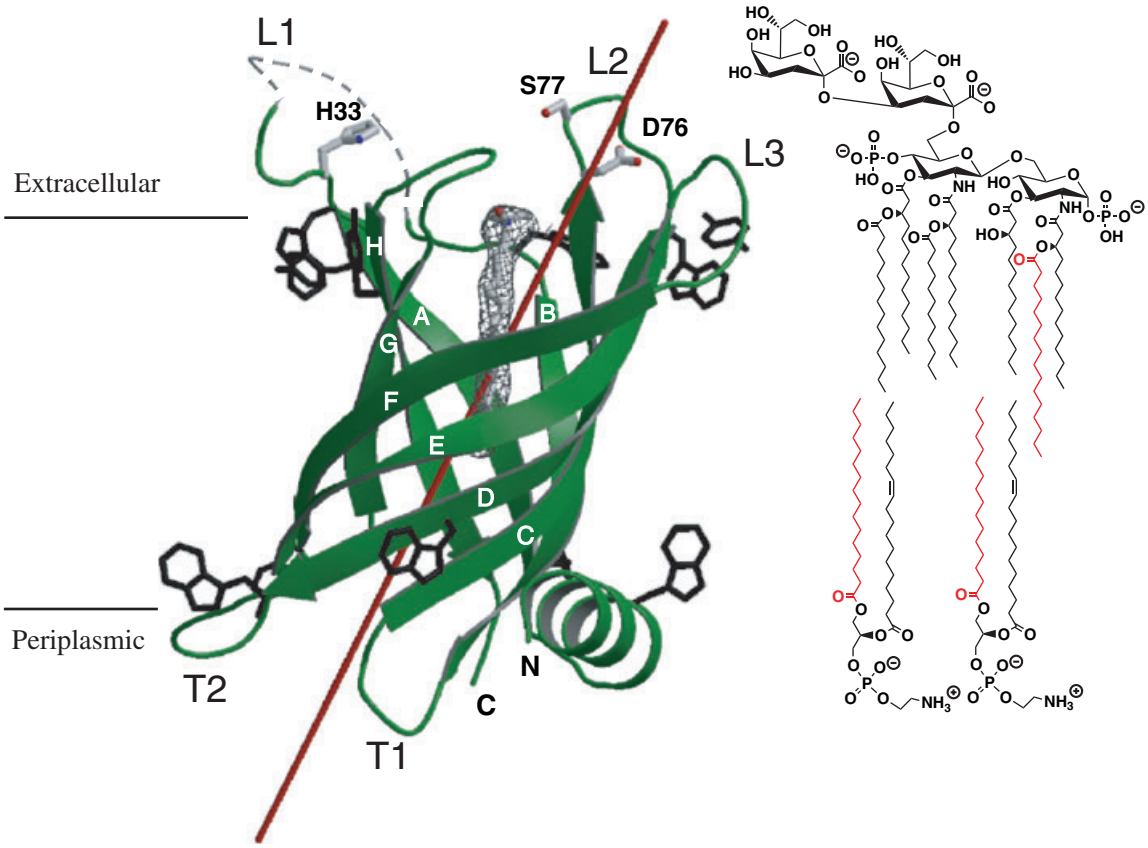

Fig. 2. Model for lipid trafficking in the control of PagP-catalysed endotoxin palmitoylation in OMs. The PagP crystal structure reveals an 8-stranded antiparallel β-barrel preceded by an amino-terminal amphipathic α-helix. A lauroyldimethylamine-*N*-oxide detergent molecule bound inside the β-barrel in the outer leaflet-exposed region defines the hydrocarbon ruler (shown in grey mesh). Possible routes for lateral diffusion of lipid substrates to the β-barrel interior are apparent in the outer leaflet between β-strands A and B at the rear of the model, and between β-strands F and G near the front. Access between β-strands A and H is also possible. The approximate position of the disordered extracellular loop L1 is indicated by a curved grey line; the ordered loops L2 and L3 and the periplasmic turns T1 and T2 are also indicated. Amino acid residues implicated in catalysis, including Ser77, Asp76 and His33, are located on the extracellular side facing the β-barrel interior. Tyrosine and tryptophan residues in the aromatic belts demarcate the membrane interfaces, which are only apparent when the barrel axis is tilted by 25° with respect to the membrane normal. The necessary steps for phospholipid trafficking to the PagP active site include destabilization of lateral LPS–LPS interactions by the removal of Mg^{2+}-ions that bridge negative charges, phospholipid translocation into the outer leaflet, and MsbA-mediated transport of phospholipids from their source of biosynthesis in the inner membrane to the OM inner leaflet. (Reprinted by permission of *EMBO J*, Ahn *et al*., 2004; copyright 2004 Macmillan Publishers. http://embojournal.npgjournals.com/).

particular detergents among those that support a native structure also support PagP activity. This enigma is now understood because those detergents that effectively mimic an acyl chain appear to function as competitive inhibitors (Ahn *et al*., 2004). The study of PagP differential dynamics using inhibitory and non-inhibitory detergent analogues revealed a conformational transition in the non-hydrogen-bonded region around the L1 loop that might reflect a structural cycle as lipids transit between the hydrocarbon ruler and the OM (Hwang *et al*., 2004). The dynamic R-state presumably facilitates the lateral diffusion of substrates and products in and out of the central cavity, while rigidification of the L1 loop in the T-state is likely required to form a catalytically competent orientation of amino acid functional groups. Such large-scale conformational transitions are commonly encountered in the catalytic cycles of the known integral membrane protein structures, including those of ion channels (Jiang *et al*., 2002; Perozo and Rees, 2003), active transporters (Chang, 2003; Abramson *et al*., 2004) and signal transducers (Kim *et al*., 2003).

Outer membrane lipid gymnastics

The clear alignment of the PagP active site with the OM outer leaflet creates an important topological problem for the enzyme. How does PagP access phospholipids if OM lipid asymmetry is maintained? LPS is located exclusively in the OM outer leaflet while phospholipids are normally restricted to the inner leaflet (Kamio and Nikaido, 1976). The asymmetric organization of OM lipids depends on divalent cations, which bridge negatively charged groups

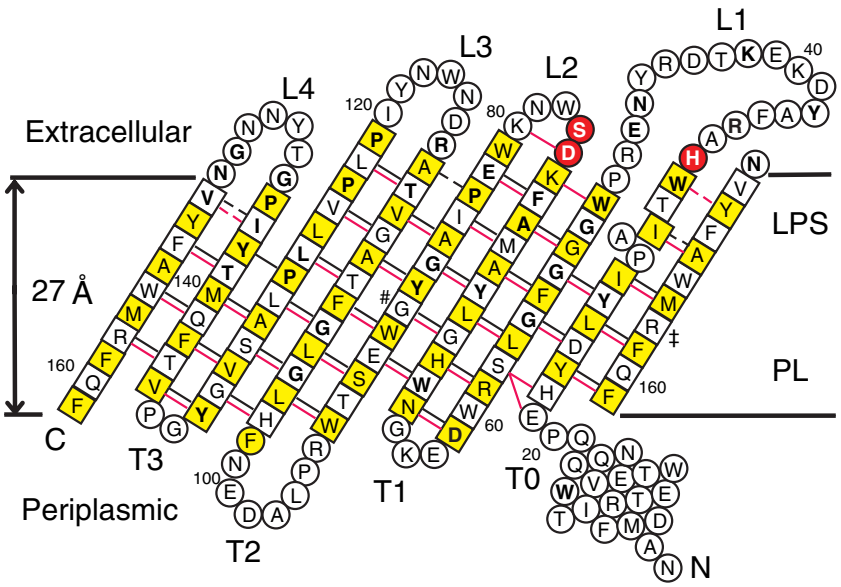

Fig. 3. Topology model of the PagP β-barrel determined by NMR in octylglucoside (OG) and dodecylphosphocholine (DPC) detergents. Slowly exchanging backbone amides are shown with black lines and HN–HN NOE connectivities are shown in red. Dashed lines indicate observations made for PagP-OG but not PagP-DPC, due to conformational exchange broadening in the latter case. The presence of the β-bulge with the extension of strand A to Trp32 is based on NOE data from PagP-OG. Invariant residues in all PagP homologues are identified in bold type. Residues in squares are part of β-strands. Residues in yellow squares have side-chains facing the membrane bilayer, while white squares indicate side-chains lining the interior of the β-barrel. The three residues important for catalytic activity as determined by site-specific mutagenesis, His33, Asp76 and Ser77, are shown on a red background. The *Y. pestis* homologue contains a premature stop codon that truncates the carboxyl-terminal three amino acid residues (‡). Gly88 lining the floor of the hydrocarbon ruler is also indicated (#), and is substituted by Ala in *Legionella* species. (Adapted from Hwang *et al*., 2002).

between neighbouring LPS molecules (Schindler and Osborn, 1979). Chelating agents such as EDTA can strip a fraction of LPS from the bacterial surface (Leive, 1974), and a large body of evidence indicates that EDTA promotes the migration of phospholipids into the OM outer leaflet (Nikaido and Vaara, 1985; Nikaido, 2003). Indeed, brief treatment of cells with EDTA induces lipid A palmitoylation through a process that occurs more rapidly than induction of the *pagP* promoter, and independently of *de novo* protein synthesis (Jia *et al*., 2004).

Continued phospholipid translocation to the OM outer leaflet is expected to create a vacuum in the inner leaflet. How are phospholipids replenished in the inner leaflet? The trafficking of phospholipids from the inner membrane to the OM is controlled by the ATP-binding cassette transporter MsbA (Doerrler *et al*., 2001; 2004). As expected, lipid A palmitoylation induced by EDTA *in vivo* requires functional MsbA (Jia *et al*., 2004). This finding suggests that PagP activity *in vivo* should require ATP for lipid trafficking (Doerrler and Raetz, 2002) even though the PagP transesterification reaction conserves the pre-existing bond energy in the phospholipid donor and, thus, does not require an exogenous energy source (Rando, 1991). A suspected role for the amino-terminal amphipathic α-helix in lipid translocation from the inner to outer leaflet of the OM was ruled out by showing that deletion of most of the helix did not adversely affect activity *in vivo* (Bishop *et al*., 1998; Jia *et al*., 2004). The *sn*-1-lysophospholipid by-product can be transported back to the inner membrane and re-acylated (Hsu *et al*., 1989) before it slowly rearranges spontaneously into the more stable *sn*-2 regioisomer (Pluckthun and Dennis, 1982).

We propose that PagP remains dormant in OMs until lipid asymmetry is broken under conditions of divalent cation limitation, which is consistent with a role for the Mg^{2+}-sensing PhoP/PhoQ system in controlling *pagP* gene expression (Fig. 2). The delay in responding to assaults on the integrity of the OM through signal transduction might necessitate an additional mechanism that responds instantaneously to changes in OM lipid organization. In this way, PagP becomes exquisitely sensitive to the divalent cations that are an integral component of OM structure. Any accumulation of phospholipids in the OM outer leaflet should be directly reported by increased lipid A palmitoylation provided that PagP expression levels and activity are taken into account. PagP could be used as a probe to answer some long-standing fundamental questions pertaining to the effects of CAMPs on OM lipids, the trafficking of lipids between the two membrane systems of Gram-negative bacteria (Jones and Osborn, 1977; Tefsen *et al*., 2005), and the mechanisms responsible for determining OM lipid asymmetry and the associated per-

meability barrier to antibiotics (Braun and Silhavy, 2002; Bos *et al*., 2004).

Towards a catalytic mechanism

Despite the wealth of information about PagP structure and dynamics, surprisingly little is known about the mechanism of catalysis. As expected for an enzyme that responds to Mg^{2+}-limitation, PagP activity is independent of divalent cations (Brozek *et al*., 1987; Bishop *et al*., 2000). The putative catalytic amino acid residues project their side-chains towards the barrel interior and are positioned above the hydrocarbon ruler. The requirement for invariant His33, Asp76 and Ser77 for catalysis (Hwang *et al*., 2002) might suggest that PagP utilizes an acyl-enzyme mechanism characteristic of known serine esterases. However, the putative active site residues are not organized into a catalytic triad that could enhance the nucleophilic character of Ser77 (Ahn *et al*., 2004) and we find that classical serine esterase inhibitors are ineffective against PagP (E.I. Lo and R.E. Bishop, unpublished). PagP exhibits slow phospholipase activity in the absence of a lipid A acceptor and various alcohols can substitute as acyl acceptors for lipid A *in vitro* (Bishop and Raetz, 2000). The natural abundance of LPS in the OM probably has not provided the selective pressure needed to achieve a high specificity in PagP for acyl acceptors. Lys42 in the L1 loop might play a role in LPS recognition, because the Lys42Ala mutant can still function as a phospholipase even though it cannot palmitoylate lipid A in certain detergent systems (E.I. Lo and R.E. Bishop, unpublished). PagP probably does not function as a phospholipase *in vivo* (Hardaway and Buller, 1979), and an induced-fit mechanism might help to shield the phospholipid at the active site from hydrolytic water molecules. The presence of two non-hydrogen-bonded regions that could provide simultaneous access for both substrates to the β-barrel interior raises the distinct possibility that PagP catalysis proceeds through the formation of a ternary complex. Such a mechanism could promote the direct transfer of the palmitoyl group from the phospholipid donor to the lipid A acceptor without the formation of an acyl-enzyme intermediate, but the detailed mechanism of PagP catalysis remains to be elucidated. Efforts to develop PagP inhibitors are now being motivated by recent observations that the enzyme plays an important role in the establishment of certain bacterial infections.

Pathogenesis of *Legionella pneumophila*

Shortly after *pagP* was first associated with CAMP resistance in *Salmonella*, a *pagP* homologue from *Legionella pneumophila* was identified as *rcp* (resistance to cationic antimicrobial peptides) (Robey *et al*., 2001). The *Legionella rcp*$^-$ phenotype is induced by growth in low-Mg^{2+} medium, but a role for the PhoP/PhoQ system in the pathogenesis of this organism remains to be established. The *rcp* gene was shown to be needed for intracellular survival in amoeba and macrophage models of Legionnaire's disease, and *rcp* mutant bacteria displayed a defect in colonization of the mouse lung. This study provided the first demonstration that *pagP* is required for intracellular infection and virulence.

It remains to be determined whether *Legionella* lipid A is actually modified by a linear 16-carbon palmitate chain. The unusual fatty acid profiles of *Legionella* lipids show them to be dominated by ω-branched acyl chains (Zahringer *et al*., 1995). Interestingly, the PagP hydrocarbon ruler is lined at its floor by a glycine residue in all homologues known to incorporate palmitate into lipid A (Ahn *et al*., 2004). Gly88 substitutions in *E. coli* PagP cause a preference for shorter acyl chains that corresponds with the length of the substituted amino acid side-chain. For example, the Gly88Ala substitution causes a preference for a linear acyl chain of 15-carbon atoms. In *Legionella*, the residue that corresponds to Gly88 is, in fact, an alanine (Fig. 3). Perhaps *Legionella* PagP is adapted to employ a 16-carbon ω-branched acyl chain, which is expected to have an extended length equivalent to that of its 15-carbon linear counterpart. The positional specificity for the hydroxyacyl chain in the lipid A disaccharide also remains to be determined for *Legionella* PagP.

The pathogenic bordetellae

PagP incorporates a palmitate chain into the lipid A of *Bordetella bronchiseptica*, a respiratory pathogen of mammals including dogs, rabbits, pigs and immunosuppressed or elderly people (Preston *et al*., 2003). Interestingly, *Bordetella* PagP incorporates a palmitate chain into lipid A on the distal glucosamine sugar at the 3′ position, where the hydroxyl group of the primary hydroxyacyl chain is not already occupied by a secondary acyl chain as found in the enterobacterial lipid As. It will be interesting to identify the molecular basis of PagP specificity for either the 2 position, in the enterobacterial lipid As, or the 3′ position, in *Bordetella* lipid A, because regulated acylation at the 3′ position appears to be important for *Bordetella* pathogenesis.

Recent findings indicate that *B. bronchiseptica pagP*, which is not required for initial colonization of the mouse respiratory tract, is required 7 days after colonization for persistence of the infection (Preston *et al*., 2003). These results were later corroborated by the observation that *B. bronchiseptica pagP* provides resistance to antibody-mediated complement lysis (Pilione *et al*., 2004). Apparently, prolonged infection activates the adaptive immune system and leads to the production of antibodies, which opsonize the bacteria for recruitment of the membrane-

attack complex of serum complement. The *pagP* promoter is activated by the BvgA/BvgS two-component virulence signal transduction pathway, which senses the host environment during *Bordetella* infection (Preston *et al*., 2003). Although a Bvg-regulated *pagP* homologue is reported to be present in *B. parapertussis*, it appears that the *pagP* promoter in *B. pertussis*, the causative agent of whooping cough in children, has been inactivated by the insertion of a transposable genetic element. Comparative genomics indicate that the acquisition of transposable genetic elements was important during the evolution of *B. parapertussis* and *B. pertussis* from a *B. bronchiseptica*-like ancestor (Parkhill *et al*., 2003; Preston *et al*., 2004). It will be interesting to determine whether *pagP* inactivation in *B. pertussis* is part of the evolution of this organism's specificity for human hosts.

The pathogenic yersiniae

The genus *Yersinia* includes the enteropathogenic species *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*, in addition to the highly virulent causative agent of bubonic and pneumonic plague, *Yersinia pestis* (Wren, 2003). A very recent evolutionary adaptation of *Y. pestis* from *Y. pseudotuberculosis* is indicated by the remarkable genetic similarity that exists between these two organisms. It appears that *Y. pestis* adaptation has been driven primarily through the loss of gene function, as numerous pseudogenes have been identified in *Y. pestis* that correspond with fully functional homologues in *Y. pseudotuberculosis* (Chain *et al*. 2004). Current evidence indicates that lipid A can be modified with a palmitate chain both in *Y. enterocolitica* and in *Y. pseudotuberculosis*, but not in *Y. pestis* (Rebeil *et al*., 2004) although an unsaturated 16-carbon acyl chain has been observed (Aussel *et al*., 2000). The *Y. pestis pagP* is very likely a pseudogene (see below), suggesting that *pagP* inactivation might have played a role in the evolution of *Y. pestis* virulence. It will be interesting to determine whether *pagP* contributes to the ability of *Y. pseudotuberculosis* and *Y. enterocolitica* to cross the intestinal epithelium after infection by the oral route. *Y. pestis* pathogenesis differs from that of the enteropathogenic species because it is directly injected into the blood stream by its flea vector (bubonic plague) or inhaled from aerosols (pneumonic plague).

The pathogenic yersiniae control virulence mechanisms partly by sensing a growth temperature shift from the external environment temperature to the body temperature of the mammalian host. Indeed, lipid A palmitoylation is strongly induced in *Y. pseudotuberculosis* by a shift in growth temperature from 21°C to 37°C (Rebeil *et al*., 2004). Palmitoylation is also induced in lipid A isolated from *Y. enterocolitica* grown under Mg^{2+}-limited conditions (Guo *et al*., 1998). However, palmitate appears to be absent from *Y. pestis* lipid A under all conditions, despite the fact that *Y. pseudotuberculosis* and *Y. pestis pagP* homologues share 99% identity at the DNA level. The only difference is the presence in *Y. pestis* of a G to A transition mutation that converts the codon for Trp200 (TGG) into an amber stop codon (TAG). Like all other PagP homologues, the amino acid sequences of *Y. pseudotuberculosis* and *Y. enterocolitica* PagP terminate three codons further downstream from this premature stop codon in *Y. pestis*, which leaves its PagP truncated by a deletion of the last three amino acid residues (Fig. 3). The possibility that the premature stop codon in *Y. pestis pagP* results from a sequencing error can be ruled out by its presence in three independently sequenced plague genomes.

The close proximity of the *Y. pestis pagP* mutation to the 3′ end of the gene gives the impression that the encoded protein is largely intact. However, inspection of the *E. coli* PagP structure implicates the last three amino acids in the formation of hydrogen bonds that close the first and final β-strands into a β-barrel in the OM (Fig. 3). These last three residues are expected to be particularly important in PagP, because the β-bulge in the first β-strand changes the registration of hydrogen bonding with the final β-strand and causes sensitivity of the outer leaflet hydrogen bonds to the detergent environment. Consequently, the last three amino acids in the PagP sequence contribute two key hydrogen bonds in the inner leaflet-exposed region that close the β-barrel structure. The absence of these three residues likely exposes the carboxyl-terminus and polar β-barrel interior region unfavourably to the phospholipid hydrocarbon chains in the OM inner leaflet. Additionally, most PagP homologues, and many outer membrane proteins in general, possess a carboxyl-terminal phenylalanine residue, which has been shown to be critical for correct OM assembly in studies of the PhoE porin (Struyve *et al*., 1991; de Cock *et al*., 1997). It seems highly unlikely that the truncated PagP molecule encoded by *Y. pestis* is capable of folding into a β-barrel in the OM.

The expression of such a defective PagP protein might require that *Y. pestis* be capable of activating proteases needed to remove misfolded proteins from the periplasmic space. The periplasmic accumulation of misfolded OM proteins can be lethal in the absence of a key periplasmic protease DegP (Misra *et al*., 2000). An extracellular function sigma factor σ^E, present in *Y. pestis*, controls transcription of genes like *degP* that are needed to respond to cell envelope stress (Alba and Gross, 2004). The conserved carboxyl-terminal peptide sequences of misfolded OM proteins can trigger the σ^E response by activating the DegS protease (Walsh *et al*., 2003), so it is unclear whether the truncated *Y. pestis* PagP could effectively signal its misfolded state. Interestingly, a recent study in *E. coli* shows that PagP-catalysed addition of palmitate to

lipid A is associated with the activation of σ^E, suggesting that palmitoylated lipid A itself can initiate signal transduction of the cell envelope stress response (Tam and Missiakas, 2005). Given that membrane perturbation can elicit the palmitoylation of lipid A (Jia *et al*., 2004), it will be interesting to learn whether or not the activation of σ^E contributes to the restoration of OM lipid asymmetry.

Erwinia and *Photorhabdus* pathogenesis

Closely related to the enterobacterial genus *Yersinia* are the plant pathogens known as the erwiniae and an insect pathogen known as *Photorhabdus luminescens*, which resides symbiotically in the gut of nematodes that employ these bacteria to feed on insects. PagP homologues are clearly apparent in the genomes of *Erwinia chrysanthemi* and *E. carotovora*, and the lipid A of *E. carotovora* has been shown to possess a structure like that of other enterobacterial lipid As, including a palmitate chain at position 2 (Fukuoka *et al*., 2001). The *P. luminescens pagP* gene is induced in response to low-Mg^{2+} growth conditions but independently of the PhoP/PhoQ system, indicating that another Mg^{2+}-sensing regulatory mechanism exists in this organism (Derzelle *et al*., 2004). Both the plant and invertebrate hosts possess elaborate innate immune defence mechanisms, suggesting that PagP in the erwiniae and *P. luminescens* might contribute to the virulence of these pathogens.

PagP in *Methylobacillus flagellatus*

An exclusive role for PagP in bacterial pathogenesis could be ruled out if it were found to be present in a non-pathogenic bacterium. The most recent PagP homologue to be identified is in the genome of *Methylobacillus flagellatus*, an obligate methylotroph not reported to have a pathogenic lifestyle, although it produces extracellular polysaccharides, a frequently observed characteristic of symbiotic and pathogenic bacteria (Yoshida *et al*., 2003). This discovery raises the possibility that PagP might exert a physiological role independent of pathogenesis. Perhaps PagP helps maintain the OM permeability barrier in response to hostile environmental conditions. Given the current number of sequenced Gram-negative genomes, it is still remarkable that *pagP* homologues are distributed narrowly among a small group of mostly pathogenic Gram-negative bacteria.

Evolutionary origins

The lipid A of *Pseudomonas aeruginosa* is found to be modified by a palmitate chain in response to activation of the PhoP/PhoQ system and when bacteria are isolated from the lungs of cystic fibrosis patients, but not when bacteria are isolated from chronic non-cystic fibrosis lung infections (Ernst *et al*., 1999). The palmitate chain in *P. aeruginosa* lipid A is added to the 3′ position, as in *Bordetella* lipid A, suggesting that a PagP enzyme could be involved. However, a different enzyme might be responsible because no *pagP* homologue is apparent in the completely sequenced *P. aeruginosa* genome (Stover *et al*., 2000). Preliminary reports indicate that *P. aeruginosa* lipid A palmitoyltransferase activity, like that of PagP, is independent of the acyl carrier protein substrates that are characteristically used in secondary acylation with laurate or myristate chains (Raetz and Whitfield, 2002; Trent, 2004). It will be interesting to learn whether the responsible enzyme represents a highly divergent PagP homologue or a case of convergent evolution.

A final point of interest lies in the striking structural similarity between PagP and a group of generally soluble lipid-binding proteins known as lipocalins (Ahn *et al*., 2004). Like PagP, the lipocalins are 8-stranded antiparallel β-barrels with a deep lipid-binding pocket at one end of the barrel and a flanking α-helix at the other end. Is it truly possible that an integral membrane protein could share a common ancestor with a soluble globular domain? Lipocalins are widely distributed among eukaryotes, but seem to be restricted to those bacteria that possess β-barrel membrane proteins (Bishop, 2000). Strikingly, the majority of bacterial lipocalins are anchored in OMs as lipoproteins (Bishop *et al*., 1995). OM β-barrel domains should be suitably adapted to occupy both soluble and membrane-bound conformations because both environments are encountered during the OM assembly process, which depends in part on periplasmic chaperones, such as Skp, and the OMP85 complex in the OM (Bos and Tommassen, 2004; Tamm *et al*., 2004; Voulhoux and Tommassen, 2004). Therefore, the hypothesis that PagP and lipocalins share a common ancestor cannot be excluded.

Conclusions

Natural variations in the structure and function of lipid A reflect the known diversity of bacterial survival strategies and provide us with useful tools for the treatment of infectious diseases. Lipid A structures that function as endotoxin antagonists are currently being developed for the treatment of septic shock (Hawkins *et al*., 2004; Stover *et al*., 2004). The number and length of acyl chains are critical factors in these studies. Additionally, detoxified lipid A derivatives can provide adjuvants for vaccines. Removal from hepta-acylated lipid A of phosphate at position 1 and *R*-3-hydroxymyristate at position 3 affords the most active congener of the monophosphoryl lipid A vaccine adjuvant (Persing *et al*., 2002). Given that PagP displays a broad specificity for lipid A acceptors (Bishop *et al*., 2000) and contains a stringent hydrocarbon ruler that is amenable to

protein engineering (Ahn *et al*., 2004), this robust enzyme might be useful for the synthesis of lipid A derivatives that function as endotoxin antagonists or as vaccine adjuvants. Additionally, PagP is already proving itself to be useful as a probe to monitor OM lipid asymmetry (Jia *et al*., 2004) and in other studies of Gram-negative cell envelope biogenesis (Tefsen *et al*., 2005). Future investigation of the PagP catalytic mechanism promises to reveal novel strategies to inhibit PagP in order to eliminate bacterial infections that depend on this fascinating enzyme.

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